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Chromosomal insertion of foreign (adenovirus type 12, plasmid, or bacteriophage λ) DNA is associated with enhanced methylation of cellular DNA segments

(DNA methylation/transfection/Ad12-transformed hamster cells/Ad12-induced hamster tumors/intracisternal A particle)

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ABSTRACT **Insertion of foreign DNA into an established mammalian genome can extensively alter the patterns of cellular DNA methylation. Adenovirus type 12 (Ad12)-transformed hamster cells, Ad12-induced hamster tumor cells, or hamster cells carrying integrated DNA of bacteriophage λ were used as model systems. DNA methylation levels were examined by cleaving cellular DNA with *Hpa* II, *Msp* I, or *Hha* I, followed by Southern blot hybridization with ^{32}P-labeled, randomly selected cellular DNA probes. For several, but not all, cellular DNA segments investigated, extensive increases in DNA methylation were found in comparison with the methylation patterns in BHK21 or primary Syrian hamster cells. In eight different Ad12-induced hamster tumors, moderate increases in DNA methylation were seen. Increased methylation of cellular genes was also documented in two hamster cell lines with integrated Ad12 DNA without the Ad12-transformed phenotype, in one cloned BHK21 cell line with integrated plasmid DNA, and in at least three cloned BHK21 cell lines with integrated λ DNA. By fluorescent *in situ* hybridization, the cellular hybridization probes were located to different hamster chromosomes. The endogenous intracisternal A particle genomes showed a striking distribution on many hamster chromosomes, frequently on their short arms. When BHK21 hamster cells were abortively infected with Ad12, increases in cellular DNA methylation were not seen. Thus, Ad12 early gene products were not directly involved in increasing cellular DNA methylation. We attribute the alterations in cellular DNA methylation, at least in part, to the insertion of foreign DNA. Can alterations in the methylation profiles of hamster cellular DNA contribute to the generation of the oncogenic phenotype?**

Adenovirus type 12 (Ad12) is oncogenic in neonate hamsters (1, 2), and the viral genome can chromosomally integrate into the genome of hamster cells (3–7). Ad12 DNA insertion is not site-specific; transcriptionally active cellular DNA sequences seem to be preferred targets (8, 9). The molecular mechanism of Ad12 DNA insertional recombination is akin to nonhomologous recombination, as studied in a cell-free system (10, 11).

We have pursued the possibility that foreign (Ad12) DNA insertion contributes to the process of tumorigenic transformation by Ad12. The conventional notion of insertional mutagenesis disrupting cellular DNA at the immediate locus of foreign DNA integration has been extended to the concept that cellular gene activity could be affected at many different cellular sites close to or remote from the locus of foreign DNA insertion due to changes in DNA methylation patterns (5, 6, 12).

In Ad12-transformed hamster cells, in Ad12-induced hamster tumor cells, and in hamster cells carrying integrated Ad12 genomes, integrated plasmid, or bacteriophage λ DNA without transformed phenotype, the methylation of several cellular DNA segments is markedly enhanced in comparison with BHK21 or primary hamster cells. The randomly selected hamster cellular DNA probes used in methylation analyses have been localized by fluorescent *in situ* hybridization (FISH) to different hamster chromosomes that do not carry Ad12 DNA. Thus, integration of foreign DNA in hamster cells exerts a distinct trans effect on the methylation of several cellular DNA segments located on different chromosomes.

MATERIALS AND METHODS

Ad12-Transformed Cell Lines, Ad12-Induced Tumors, and Hamster Cells Carrying Plasmid or Bacteriophage λ DNA. The derivations of cell lines (see Table 1) and the conditions of their cultivation have been described (9, 13–15). Cell line T637 carried in an integrated form >20 Ad12 genome equivalents, its revertant line TR12 contained a few copies, and the TR3 cell line carried no Ad12 DNA (16). Tumors were induced in newborn Syrian hamsters (*Mesocricetus auratus*) by injecting CsCl-purified Ad12 within 24 hr after birth (1, 14). In the BHK21 cell lines H-Ad12neo2 and H-Ad12neo5 (15), Ad12 DNA or its fragments were genomically fixed after cotransfection of the pSV2-neo plasmid (17) and selection for G418 resistance. These cells had BHK21 morphology and not that of Ad12-transformed cells. Plasmid DNA or the DNA of bacteriophage λ cI857 was also genomically integrated by cotransfection (15).

DNA Cleavage. Cellular DNA was cleaved with the methylation-sensitive *Hha* I or *Hpa* II using 10 units or in some experiments 30 units of enzyme per μg of DNA. Standard methods of molecular biology were used (13, 14, 18–21).

FISH (22). Different RNA-free cellular DNA probes (2 μg) (see Table 1) or the *Pst* I D fragment (nt 20,885–24,053; ref. 23) of Ad12 DNA was nonradioactively labeled either with biotin 16-dUTP or with digoxigenin-dUTP (Boehringer Mannheim) by nick-translation (24), as described (G. Meyer zu Altenschildesche, P.W., H.H., S. T. Tjia, and W.D., unpublished work) using 60 ng of labeled DNA.

After hybridization and extensive washing (G. Meyer zu Altenschildesche, P.W., H.H., S. T. Tjia, and W.D., unpublished work), DNA was treated with a mixture of anti-digoxigenin–rhodamine conjugate diluted 1:4 (Boehringer Mannheim) plus avidin–fluorescein isothiocyanate (FITC) diluted 1:400 (vector A2011) in blocking solution (3% milk

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Abbreviations: Ad12, adenovirus type 12; FISH, fluorescent *in situ* hybridization; IAPI, intracisternal A particle segment I; MHC, major histocompatibility complex; p.i., postinfection; FITC, fluorescein isothiocyanate; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride.

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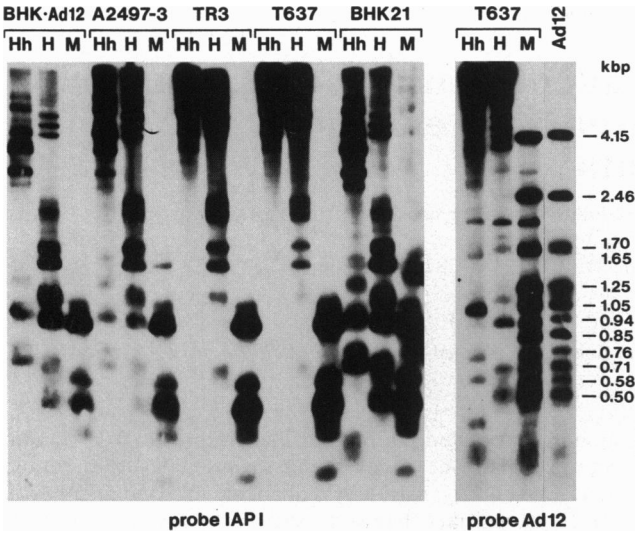

FIG. 1. Increases in DNA methylation in the IAPI DNA segment in Ad12-transformed hamster cell lines in comparison with BHK21 cells. The nuclear DNAs from cell lines as indicated were isolated by standard procedures and cleaved with *Msp* I (M), *Hpa* II (H), or *Hha* I (Hh); the fragments were separated by electrophoresis on a 1% agarose gel, transferred to a Hybond-N^+ nylon membrane, and hybridized to the ^{32}P-labeled IAPI probe (compare in Table 1). *Hha* I or *Hpa* II cleavage patterns differed strikingly for Ad12-transformed cell lines and uninfected or Ad12-infected BHK21 cells (*Left*). As a control, the same blot was freed from the IAPI probe by boiling in 0.1% SDS for 30 min, and the DNA was then hybridized with ^{32}P-labeled Ad12 DNA (*Right*, data shown only for T637 DNA). Hybridization probes are designated at bottom. At right the *Msp* I cleavage pattern of authentic Ad12 virion DNA and the sizes in kbp of the *Msp* I fragments of Ad12 DNA are indicated.

powder in 4× standard saline/citrate) at 37°C for 30 min. The slides were washed, and a 1:40 dilution of rabbit anti-sheep IgG (vector AI-6000) was added, and slides were incubated for 30 min at 37°C. After the washing, a mixture of anti-rabbit rhodamine conjugate diluted 1:4 (Boehringer Mannheim 1238-841) and biotinylated anti-avidin D diluted 1:40 (vector BA0300) was added; the slides were again incubated for 30 min at 37°C. The reaction with avidin–FITC was repeated. DNA was counterstained in propidium iodide solution (200 ng/ml) or in 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; 200 ng/ml).

The labeled karyotypes were photographed with a SC-35 type 12 camera under ultraviolet light in an Olympus BH-2 microscope that used the Olympus filterblock 61002 (DAPI/FITC/Texas Red stain).

RESULTS AND DISCUSSION

Increase of Cellular DNA Methylation in Ad12-Transformed Hamster Cell Lines. In the Ad12-transformed cell lines T637 (origin BHK21 cells), its morphological revertant cell line TR3 (25), and, to a lesser degree, in cell lines A2497-3 and HA12/7, the extent of DNA methylation at the 5′-CCGG-3′ and 5′-GCGC-3′ sequences markedly increased in the regions of the endogenous intracisternal A particle (IAP) segment I (IAPI) of the genome (Fig. 1) and in the major histocompatibility complex (MHC) class I segment I (compare with Fig. 2; see Table 1). The same DNA segments were much less methylated in BHK21 (Figs. 1 and 2) and in primary hamster cells (data not shown) because these DNA sequences were cut by *Hpa* II or *Hha* I. Changes in DNA methylation were different in each cell line and stable in revertant cell line TR3, devoid of Ad12 DNA (Figs. 1 and 2; see Table 1), and revertant TR12, which had lost most of the Ad12 DNA. An increase in DNA methylation in the MHC region (Fig. 2) and the serine proteinase gene was not observed in revertant TR12.

Cellular DNA methylation patterns were also studied after Ad12 infection of BHK21 cells because early Ad12 functions might contribute to these changes. At 30 hr postinfection (p.i.) of BHK21 cells with 70 plaque-forming units (pfu) of Ad12 per cell, or at 8, 11, or 27 days p.i. with Ad12 at 700 pfu per cell, the cellular DNA methylation patterns were unchanged (Figs. 1 and 2; see Table 1). Successful Ad12 infection of BHK21 cells was documented by the presence of Ad12 early-region E1-specific RNAs at 30 hr p.i. in RNA transfer hybridization experiments (data not shown). Expression of early Ad12 genes, which is very similar in Ad12-infected BHK21 cells and in T637 cells (26), is probably not responsible for the increase in cellular DNA methylation. Rather, foreign DNA integration and/or Ad12 transformation must have elicited these changes.

Complete cleavage by *Hpa* II or *Hha* I was ascertained (*i*) with DNA from uninfected or Ad12-infected BHK cells (Figs. 1 and 2), (*ii*) by finding the same DNA cleavage patterns for

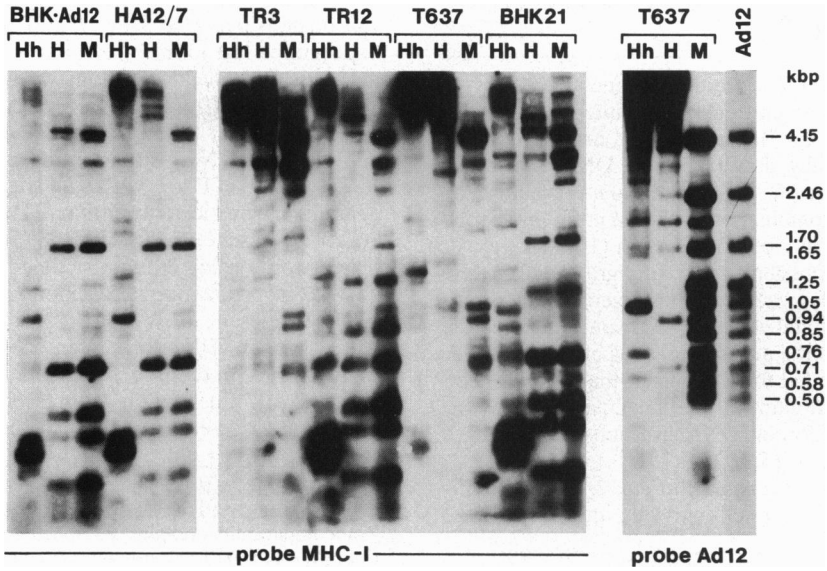

FIG. 2. Increases in DNA methylation in MHC class I, DNA segments I in Ad12-transformed hamster cell lines in comparison with BHK21 cells. For details, see the legend for Fig. 1.

Table 1. Alterations of DNA methylation patterns in cellular genes in Ad12-transformed hamster cells

Cloned hamster cell DNA*	DNA from cell type													
	T637		TR3		A2497-3		HA12/7		BHK21·Ad12 (30 hr p.i.)		H-Ad12neo5		H-Ad12neo2	
	Hpa II	*Hha* I	*Hpa* II	*Hha* I	*Hpa* II	*Hha* I	*Hpa* II	*Hha* I	*Hpa* II	*Hha* I	*Hpa* II	*Hha* I	*Hpa* II	*Hha* I
Genomic clones														
IAPI	++	++	++	++	(+)	(+)	+	+	−	−	+	+	+	(+)
IAP, II	+	+	+	+	−	−	−	−	−	−	ND		ND	
IAP, III	+	++	+	++	−	−	−	+	−	−	ND		ND	
IAP, IV	+	++	+	++	−	−	−	+	−	−	ND		ND	
MHC class I, I	++	++	+	+	(+)	(+)	(+)	−	−	−	+	(+)	(+)	(+)
MHC class I, II	+	+	+	+	+	+	−	−	−	−	ND		ND	
Ig Cμ, I	++	++	++	++	(+)	+	−	−	−	−	ND		ND	
Ig Cμ, II	++	++	+	+	(+)	(+)	−	−	−	−	(+)	(+)	(+)	(+)
ADPRT†	−	−	ND		−	−	−	−	−	−	−	−	−	−
cDNA clones														
Serine proteinase	++	++	++	++	++	++	+	+	−	−	−	−	−	−
Cytochrome P450	++	+	++	+	+	++	+	++	−	−	−	−	−	−
Asparagine synthetase	−	−	−	−	−	−	ND		−	−	−	−	−	−
Serum amyloid A	−	−	−	−	−	−	ND		−	−	−	−	−	−

BHK21 cells are a permanent hamster fibroblast cell line (29). The cleavage patterns generated with *Hpa* II (*Msp* I) or *Hha* I on BHK21 DNA and visualized by hybridization with the ^{32}P-labeled probes as indicated have served for comparisons with the cleavage patterns with the DNA from all other cell lines (reference patterns). T637 cells are an Ad12-transformed BHK21 hamster cell line (30) that contain >20 integrated copies of Ad12 DNA (13). Cell lines A2497-3 (31) and HA12/7 (32) have been generated by Ad12 transformation of primary hamster cells. These cell lines carry ≈12–18 and 2–3 integrated copies of Ad12 DNA, respectively. BHK21 cells have been infected with CsCl-purified Ad12 at a multiplicity of infection of ≈70 plaque-forming units per cell. The symbols (+), +, and ++ indicate increased levels of DNA methylation for the indicated probes in different cell lines in comparison with the reference patterns obtained with BHK21 or primary hamster DNA (for IAPI probe). The symbol − designates that levels of DNA methylation are indistinguishable from those found in BHK21 DNA. Increases in DNA methylation in a given DNA segment have been identified by its decreasing cleavability by the methylation-sensitive restriction endonuclease *Hpa* II or *Hha* I. Cleavability by *Msp* I (isoschizomer to *Hpa* II) has served as an internal control. ND, not done.

*The cloned hybridization probes have all been derived from Syrian hamster DNA, except for the ADPRT clone, which originated from Chinese hamster DNA. These clones have been described elsewhere: four segments (I–IV) of IAP DNA, an endogenous viral genome (33, 34); two segments (I, II) of MHC class I (35); two segments (I, II) from the heavy chain of immunoglobulin constant region μ (IgCμ) (36); serine proteinase (37); cytochrome P450 (38); asparagine synthetase (39); serum amyloid A (40); adenine phosphoribosyltransferase (ADPRT) (41). Of the 1.9 kbp of IAPI sequence, ≈1 kbp has been redetermined and found identical to the published (34) nucleotide sequence.

†DNA probe from Chinese hamster.

T637 cells and for Ad12-infected BHK21 cells at a 3-fold higher enzyme concentration (30 units) (Figs. 1 and 2), and (*iii*) by hybridizing the DNA on the same filters to ^{32}P-labeled Ad12 DNA (Figs. 1 and 2). The known *Hpa* II patterns of integrated Ad12 DNA in these cell lines were confirmed (Figs. 1 and 2; refs. 27, 28).

Survey of Ad12-Transformed Cells and of Ad12-Induced Tumors Using Additional Cellular Hybridization Probes. The results of similar experiments using a number of randomly selected cellular probes were summarized in Table 1. Enhanced DNA methylation was observed with some, but by no means all, cellular probes. Alterations of DNA methylation varied in different cell lines—possibly due to different sites of viral DNA integration and the different nature of cell lines. In eight different Ad12-induced hamster tumors (7, 14), moderate increases in cellular DNA methylation for MHC class I probe I and for the IAPI probe were seen (Table 2).

Increase in Cellular DNA Methylation in Cell Lines H-Ad12neo2 and H-Ad12neo5. These cell lines carried multiple integrated copies of incomplete Ad12 DNA. These cells did not express the Ad12 genome and lacked the Ad12-transformed phenotype (15). The 5′-CCGG-3′ and 5′-GCGC-3′ sites in IAP clone I, in MHC class I, and in immunoglobulin constant region μ II regions showed moderate increases in DNA methylation when compared with BHK21 DNA. In other cellular DNA regions no changes in methylation were seen (Table 1).

Increased DNA Methylation in the IAPI Segments in Cloned BHK21 Cell Lines with Integrated Bacteriophage λ or Plasmid DNA. We also analyzed several BHK21 cell lines that carried genomically integrated bacteriophage λ DNA. In BHK21-λ clones 7, 13, and 15, but less in BHK21-λ clones 21 and 24, striking increases in DNA methylation were seen in the IAPI segment (Fig. 3); similar results were observed in one BHK21 cell line with integrated plasmid DNA (data not shown). Thus, changes in cellular DNA methylation were not dependent on the Ad12-transformed phenotype. The integration of foreign (λ) DNA, at different sites in different cell lines

Table 2. Increases in DNA methylation of cellular genes in Ad12-induced hamster tumors

Cloned hamster DNA	DNA from Ad12-induced tumor							
	T181	T191	T201/2	T201/3	T211	HT5	T1111(1)	T1111(2)
IAPI	+	+	(+)	(+)	+	(+)	(+)	(+)
MHC class I, probe I	(+)	(+)	(+)	ND	(+)	+	(+)	(+)

All tumor DNAs were cleaved with *Hpa* II or *Msp* I and analyzed with the two cellular hamster DNA probes as indicated. The Ad12-induced hamster tumors T181, T191, T201/2, T201/3, T211, and HT5 are described in ref. 14; the Ad12-induced tumors T1111(1) and T1111(2) are described in ref. 42. All other designations are similar to those in the legend for Table 1. Increases in DNA methylation [+, (+)] are stated in comparison with the patterns in BHK21 DNA (Fig. 1, Table 1). ND, not done.

(FISH; data not shown), sufficed to elicit enhanced methylation in the IAP DNA segments. The integrated λ DNA was methylated.

FISH Localization of the Cellular Hybridization Probes to Chromosomes Different from Those Carrying Ad12 DNA. The >20 copies of partly rearranged Ad12 DNA in cell line T637 were located at one site on one hamster chromosome (Fig. 4*a*). The cellular retrotransposon-like element IAP, which was represented in the hamster genome in some thousand copies (33), was found under high-stringency hybridization conditions on most hamster chromosomes, frequently on their short arms (44) (Fig. 4*b*). In Fig. 4*c*, the chromosomal DNA of T637 cells was stained blue with DAPI, the IAP sequences in hamster DNA were stained red with a digoxigenin–rhodamine probe, and the integrated Ad12 genomes were stained green (arrowhead) by the FITC avidin–biotin system. These cytogenetic data then placed the IAP sequences onto many different chromosomes in the hamster genome, far remote from the site of Ad12 DNA integration.

Similar conclusions were drawn for the MHC sequences. Cytogenetic FISH analysis of the T637 hamster karyotype placed the MHC probe into a location close to the terminus of the short arm of a chromosome (Fig. 5) different from the one carrying the integrated foreign (Ad12) DNA (compare with Fig. 4*a*).

Increased Transcription of Cellular IAPI Sequences in Cell Lines T637, TR3, and A2497-3. The results of RNA transfer experiments with RNAs from BHK21, Ad12-infected BHK21 (30 hr p.i.), T637, TR3, or A2497-3 hamster cells and of hybridization to the IAPI hamster DNA probe revealed an increased level of IAPI transcription in cell lines T637, TR3, and A2497-3 (data not shown). With the MHC and immunoglobulin constant region μ hybridization probes, signals could not be detected; the serine proteinase DNA probe yielded comparable signals with RNAs from these four cell lines.

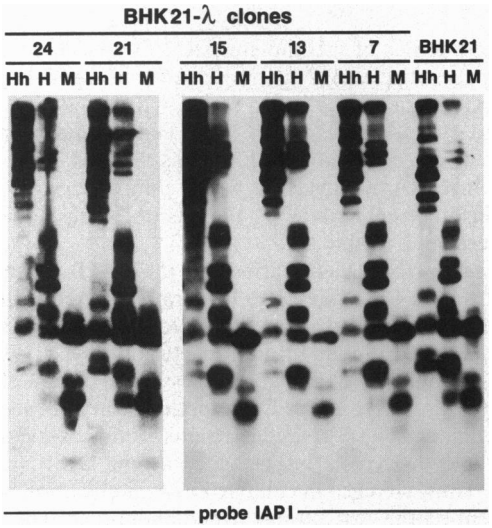

FIG. 3. Increases in DNA methylation in IAPI segments in cloned BHK21 cell lines carrying integrated λ DNA. BHK21 cells were cotransfected with λ cI857 DNA and pSV2neo DNA. G418-resistant clones were selected and recloned three times. The presence of integrated bacteriophage λ DNA was determined by cleaving DNA from clonal isolates with *Eco*RI and by Southern blot hybridization analyses with ^{32}P-labeled λ cI857 DNA as probe. Integration patterns in BHK21-λ clones 7, 13, and 15 differed but were similar in BHK21-λ clones 21 and 24 (data not shown). Changes in DNA methylation patterns in the IAPI segment were determined as described for Fig. 1. The sources of DNA were indicated. The *Msp* I (M), *Hpa* II (H), and *Hha* I (Hh) patterns of DNA from BHK21 cells served as references. Lanes BHK21 7, 13, 15 and lanes 21, 24 were derived from different electrophoresis experiments.

CONCLUSION

In Ad12-transformed hamster cells, Ad12-induced hamster tumors, in hamster cells, which carry integrated Ad12 genomes but do not show the Ad12-transformed phenotype, and in hamster cells with integrated bacteriophage λ or plasmid

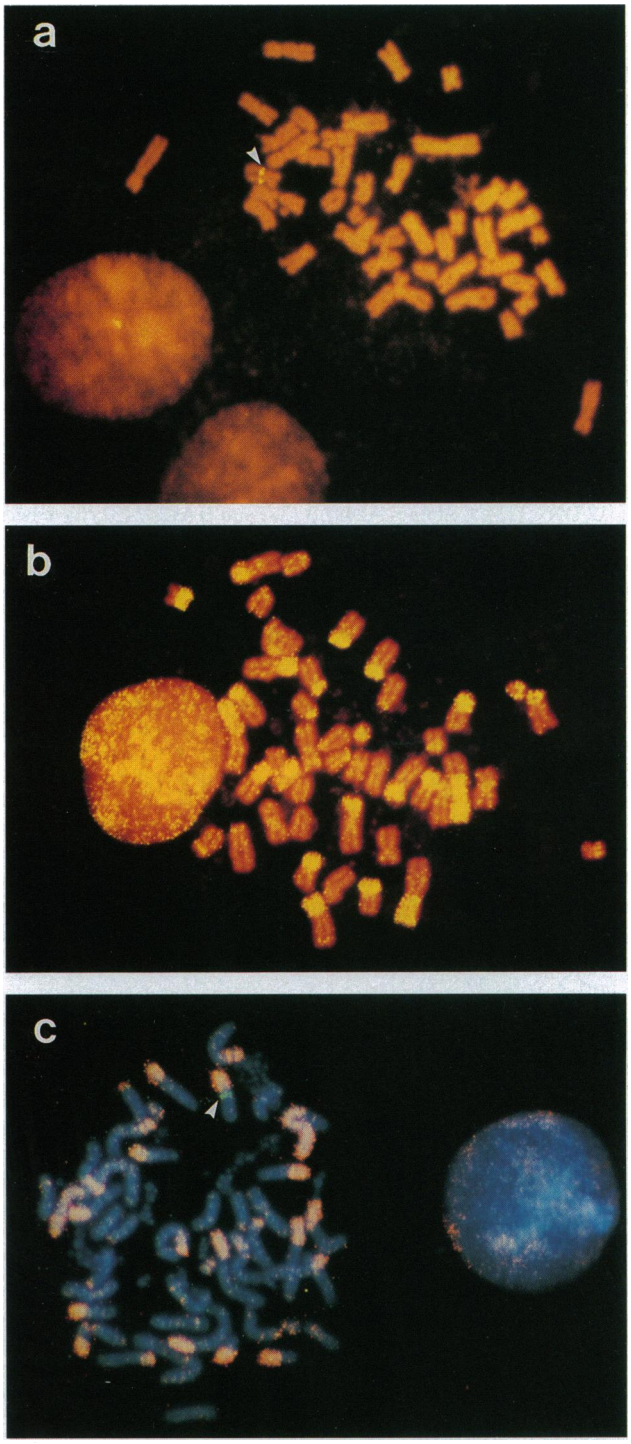

FIG. 4. FISH of spread chromosomes from the Ad12-transformed hamster cell line T637. (*a*) Biotinylated pBluescript-cloned nt 20,885–24,053 (*Pst* I-fragment D) probe of Ad12 DNA was used. (*b*) Biotinylated hamster IAPI DNA probe was used. In *a* and *b* chromosomal DNAs were counterstained with propidium iodide. (*c*) A mixture of biotinylated *Pst* I-fragment D probe of Ad12 DNA and digoxigenin-labeled IAPI cellular DNA probe was applied. In *c*, chromosomal DNA was counterstained with DAPI. Arrowheads in *a* and *c* designate Ad12 DNA.

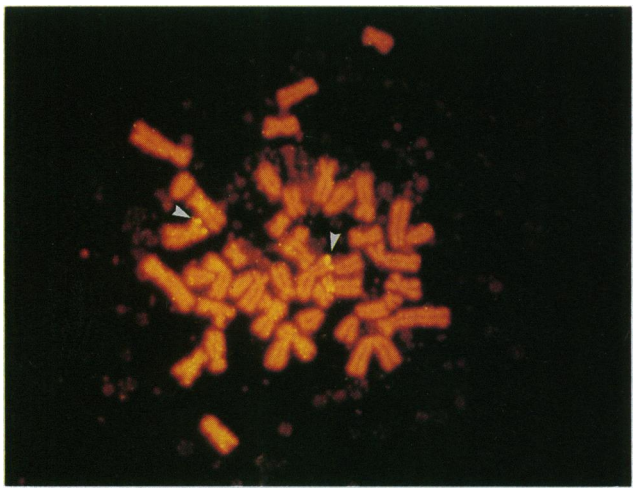

FIG. 5. FISH of spread chromosomes from Ad12-transformed hamster cell line T637 with hamster MHC class I probe I DNA. Arrowheads, MHC probe on both chromosome homologs.

DNA, the insertion of foreign DNA into the host genome can be accompanied in a cell type-specific manner by increases in DNA methylation in several cellular genes or DNA segments. These changes affect DNA segments on chromosomes different from the one on which the foreign (Ad12) genomes are integrated (Figs. 1 and 4). There are, of course, cellular DNA segments devoid of changes in DNA methylation patterns (Figs. 2, 3, and 5; Tables 1 and 2).

Methylation patterns of the IAPI DNA segment are indistinguishable between primary hamster cells and the spontaneously transformed control cell line BHK21 (29). The observed changes cannot be solely due to cellular transformation and continuous cultivation: cell line T637 has been derived from the hamster cell line BHK21 by infection with Ad12 (30). Clonal BHK21 cell lines with integrated λ DNA or plasmid DNA also show increases in cellular (IAPI) DNA methylation.

Because the patterns of expression of Ad12 early genes are very similar in Ad12-transformed hamster cells and early after the abortive infection of BHK21 hamster cells with Ad12 (26), we have investigated methylation patterns in Ad12-infected BHK21 cells at 30 hr and several weeks after infection. Changes in DNA methylation patterns in the Ad12-infected BHK21 cells have not been seen. We thus argue that the insertion of different types of foreign DNA (Ad12, λ, plasmid DNA) into a defined mammalian genome contributes to the reported increases in cellular DNA methylation. We do not yet know by what mechanism the insertion of foreign DNA can act in trans to alter cellular DNA methylation patterns.

The increases in DNA methylation of specific cellular genes are also apparent in the revertants of cell line T637, TR3, and TR12, which have lost all or most of the Ad12 genomes, respectively. The changes in cellular DNA methylation upon foreign DNA insertion are thus stable beyond the loss of integrated foreign DNA.

Many of the endogenous retrotransposon-like IAP DNA sequences are located on the short arms of a large number of hamster chromosomes (44) (Fig. 4 *b* and *c*). This unexpected observation has been made under high-stringency hybridization conditions (G. Meyer zu Altenschildesche, P.W., H.H., S. T. Tjia, and W.D., unpublished work).

The finding that cellular DNA methylation patterns are very significantly altered in virus-transformed cells and in Ad12-induced tumors raises questions as to the role such changes could play in the mechanism of viral oncogenesis. Increases in DNA methylation might be associated with the shutoff of cellular functions; decreases in DNA methylation might be associated with the activation of previously silent genes or DNA segments. We have reported that among 40 randomly selected hamster genes, 5 genes show altered transcription patterns (43). These results may also bear on work with transgenic organisms and in gene therapy.

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